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The phosphorylation state of *Saccharomyces cerevisiae*
linker histone Hho1p during entry and exit of stationary
phase

Sachin J. Somers

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Declaration

The experimental work described in this report was carried out in the Department of Molecular and Cell Biology, University of Cape Town, from February 2003 to June 2005, under the supervision of Associate Professor Hugh G. Patterson.

The results presented here are the original, unaided work of the author. Where use has been made of the work of others it is duly acknowledged in the text.

Signed by candidate

Sachin J. Somers

August 2005

Abstract

Our group has recently found that the linker histone Hho1p of *Saccharomyces cerevisiae* exhibited a significant increase in binding to chromatin during stationary phase. Because of the role of H1 in gene expression and chromatin compaction, it is essential to understand the mechanism behind this change in binding behaviour for a complete mechanistic description of gene regulation. We postulated that the phosphorylation of serine or threonine residues decrease the affinity of H1 for DNA, resulting in the dissociation of H1 from chromatin in exponential phase. We investigated this possible change in the phosphorylation state of Hho1p in yeast cells in exponential phase and in stationary phase by immunoprecipitation of Hho1p, followed by western analysis using anti-phosphoserine and anti-phosphothreonine antibodies. We found that both serine and threonine residues in Hho1p were phosphorylated, but could not detect a significant change in the phosphorylation state of these residues during entry and exit of stationary phase. To address the possibility of subtle modification changes, or alternative covalent modifications, we attempted the affinity purification of Hho1p, followed by MALDI-TOF mass spectrometric analysis. Although the MALDI-TOF approach was methodologically successful, we could not prepare sufficiently pure Hho1p sample for the study. We discuss possible reasons for the difficulty in isolating linker histone from yeast, and report methodologies that we attempted. We finally propose several alternative mechanisms that can regulate binding of H1 to chromatin, and discuss experimental approaches that can be used to address these alternatives.

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Table of Contents

Abbreviations	vii
Chapter 1: Introduction	1
1.1 Chromatin and the nucleosome	1
1.2 Chromatin fibre	3
1.3 Nucleosome dynamics	5
1.3.1 Chromatin remodeling	5
1.3.1.1 ATP-dependent remodeling complexes	5
1.3.1.2 Post-translational Modifications	7
1.4 Covalent modifications of linker histones	10
1.4.1 Linker histone phosphorylation	10
1.4.2 H1 and core histone acetylation	13
1.4.3 DNA methylation and H1	14
1.5 The Histone Code hypothesis	15
1.6 Project Aim	15
Chapter 2: Materials and Methods	17
2.1 Yeast Strains	17
2.2 Determination of the phosphorylation states of Hho1p	17
2.2.1 Growth conditions	17
2.2.2 Protein extraction	17
2.2.3 Protein concentration determination	18
2.2.4 Protein Immunoprecipitation	18
2.2.4.1 Preparing the antibody bound Sepharose	18
2.2.4.2 Binding the protein to the antibody-bound Sepharose	18

2.2.4.3 SDS-PAGE Electrophoresis	19
2.2.2.4 Western Blot	19
2.2.4.1 Stripping the membrane	20
2.3 Immuno slot/dot blotting	20
2.4 Affinity chromatography	21
2.4.1 Protein extraction for affinity chromatography	21
2.4.2 Preparing the column	21
2.4.3 Binding and eluting the protein	21
2.5 Perchloric acid extraction of Histone Hho1p	22
2.6 Sephadex CM52 purification of histones	22
2.7 MALDI-TOF mass spectrometry	23
2.7.1 Silver staining	23
2.7.2 MALDI-TOF mass spectrometry	23
Chapter 3: Results	25
3.1 Does the phosphorylation state of Hho1p change in the stationary phase?	25
3.2 Western blots of the immunoprecipitations	25
3.3 Hho1p extraction	29
3.3.1 Protein purification via the c-myc column	30
3.4 Silver stain of the samples prior to MALDI-TOF	30
3.5 MALDI-TOF mass spectrometry	32
3.6 Identification of the protein in band 1	33
3.7 Identification of the second band	35
3.8 Identification of the third band	35
3.9 Identification of the fourth band	38
3.10 Perchloric acid extraction of Hho1p	38
Chapter 4: Conclusion and Discussion	42

4.1 A model for H1 regulation of DNA function	42
4.2 Is Hho1p phosphorylated?	42
4.3 Future directions	45
Reference List	47

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Abbreviations

°C – degrees celsius

µg – micrograms

µg/ml – micrograms per millilitre

µl – microlitres

Å – Angstroms

ACF – ATP-dependant chromatin assembly and remodeling factor

ATP – adenosine triphosphate

bp – base pairs

BSA – bovine serum albumin

CHRAc – chromatin accessibility complex

DNA – deoxyribonucleic acid

ECL – enhanced chemiluminescence

GFP – green fluorescent protein

HDAC – histone deacetylase

HMG – high mobility group

ISWI – Imitation of Switch

kDa – kilo daltons

MALDI-TOF - matrix assisted laser desorption/ionization time-of-flight

MAPK – mitogen-activated protein kinase

MBD – methyl binding domain

ml – milliliters

NURD – nucleosome remodeling deacetylase

PBS – phosphate buffered saline

PIKK – phosphoinositide-3-kinase-related protein kinase

PRMT – protein arginine N-methyl transferase

PHD – plant homeo domain

RNA – ribonucleic acid

RSC – remodels the structure of chromatin

SDS – sodium dodecyl sulphate

SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis

SWI/SNF – switching/sucrose non-fermenting

TBS – Tris buffered saline

V – volts

v/v – volume by volume

w/v – weight by volume

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Chapter 1

Introduction

1.1. Chromatin and the nucleosome

In eukaryotic organisms DNA is extensively packaged into an array of nucleosomes that is further compacted to form 30nm chromatin fibers and higher-order structures. Each nucleosome consists of approximately 168bp of DNA which is spooled onto a histone octamer, consisting of two copies each of the core histones H2A, H2B, H3 and H4 (Figure 1.1). The histone pairs H2A-H2B and H3-H4 form heterodimers by extensive hydrophobic and hydrogen-bond interactions. The H3-H4 heterodimer forms a H3-H4 tetramer by a four helix bundle interaction between the C-terminal domains of H3 within each H3-H4 dimer. The H2A-H2B heterodimer binds to the H3-H4 tetramer by a similar four helix bundle interaction between the C-termini of H2B and H4. A fifth histone, the linker histone H1, binds to the outside of the nucleosome, straddling the two DNA gyres between a position close to the nucleosomal axis of two-fold symmetry, and the terminal helical turn of the nucleosomal DNA.

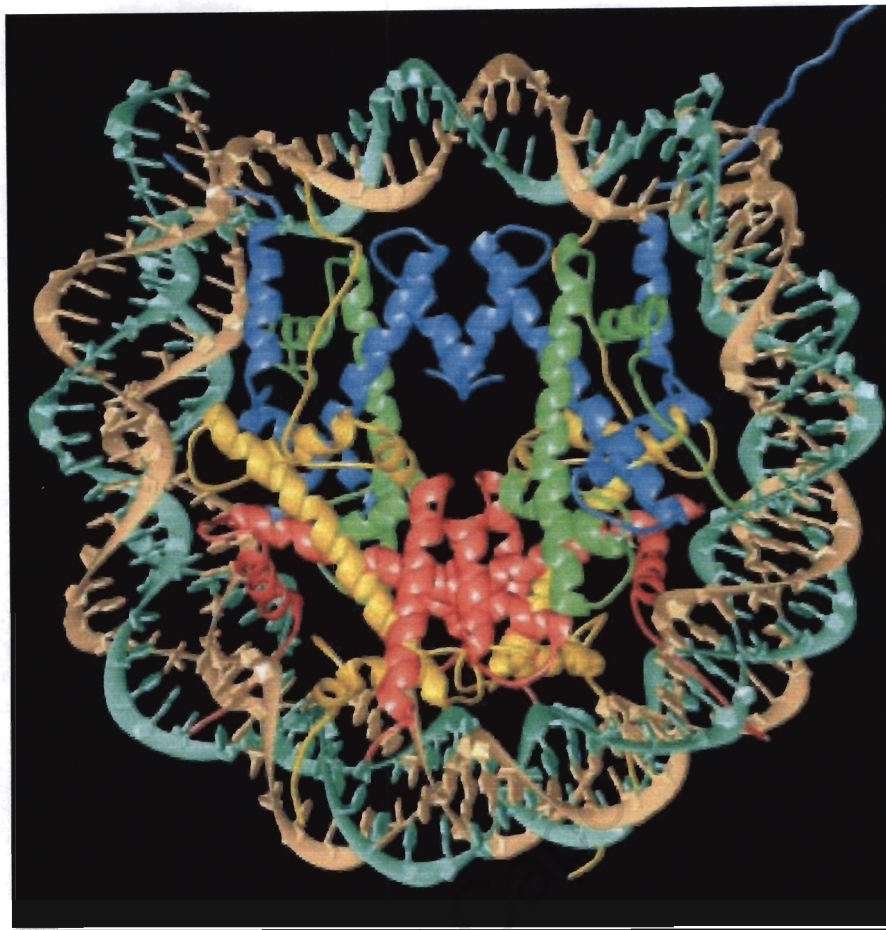


Figure 1.1 Crystal structure of the nucleosome core particle as visualized at 2.8 Å resolution. A ribbon diagram representing 146bp DNA (illustrated in brown and turquoise) which is coiled around the core histone octamer (The following colours used to differentiate between the histones: blue for H3, green for H4, yellow for H2A and red for H2B) (Luger et al., 1997).

Each of the core histones possess a structurally conserved motif, known as the histone fold. The histone fold consists of three α -helices separated by two loops, to form an $\alpha 1$ -L1- $\alpha 2$ -L2- $\alpha 3$ arrangement (Ramakrishnan, 1997). The structure of linker histone H1 or the extreme H1 variant, H5, is different from that of the core histones. It consists of a central globular domain, formed by a single-winged helix fold, that is flanked by short N-terminal tail and a lysine-rich C-terminal tails (Ramakrishnan, 1997). *Saccharomyces cerevisiae* was not known to possess histone H1 until 1998, when Landsman published findings of a putative H1 which was encoded by *HHO1* (Landsman, 1996). The protein was termed Hho1p.

Subsequent research into the phenotype of *HHO1* did not yield any phenotype for the gene (Ushinsky et al., 1997, Patterson et al., 1998). It was recently shown that Hho1p possessed two globular domain separated by a lysine-rich interglobular region (Ali and Thomas, 2004). The two H1 members from *Tetrahymena* do not possess globular domains (Wu et al., 1986).

1.2. The 30nm chromatin fibre

The structural detail of the 30nm fiber has been the subject of extensive debate. Two major classes of models have been proposed (Figure 1.2). In the solenoid model, the nucleosomal array is proposed to be folded into a single-start contact superhelix with approximately 6 nucleosome per turn, and a superhelix pitch of approximately 110Å (Finch et al., 1977). In the two-start model alternating nucleosomes in the array are in contact, and stacked in two helices, with the inter-nucleosome linker DNA connecting adjacent nucleosomes through the fiber center, or along a path parallel to the fiber axis, connecting nucleosomes on neighbouring turns of the superhelix. The solenoid model was long held in favour (Finch et al., 1997; Widom and Klug, 1985). Recently Richmond and co-workers showed, using nucleosome arrays containing mutated core histones that could be crosslinked via disulfide bridges, that the chromatin fiber was a two start helix (Richmond and Davey, 2003). In addition, they crystallized a tetranucleosome, consisting of the Widom '601' sequence, in which it was shown that at 9Å resolution that DNA was coiled into a zig-zag fashion, consistent with a two-start helix (Schalch et al., 2005).

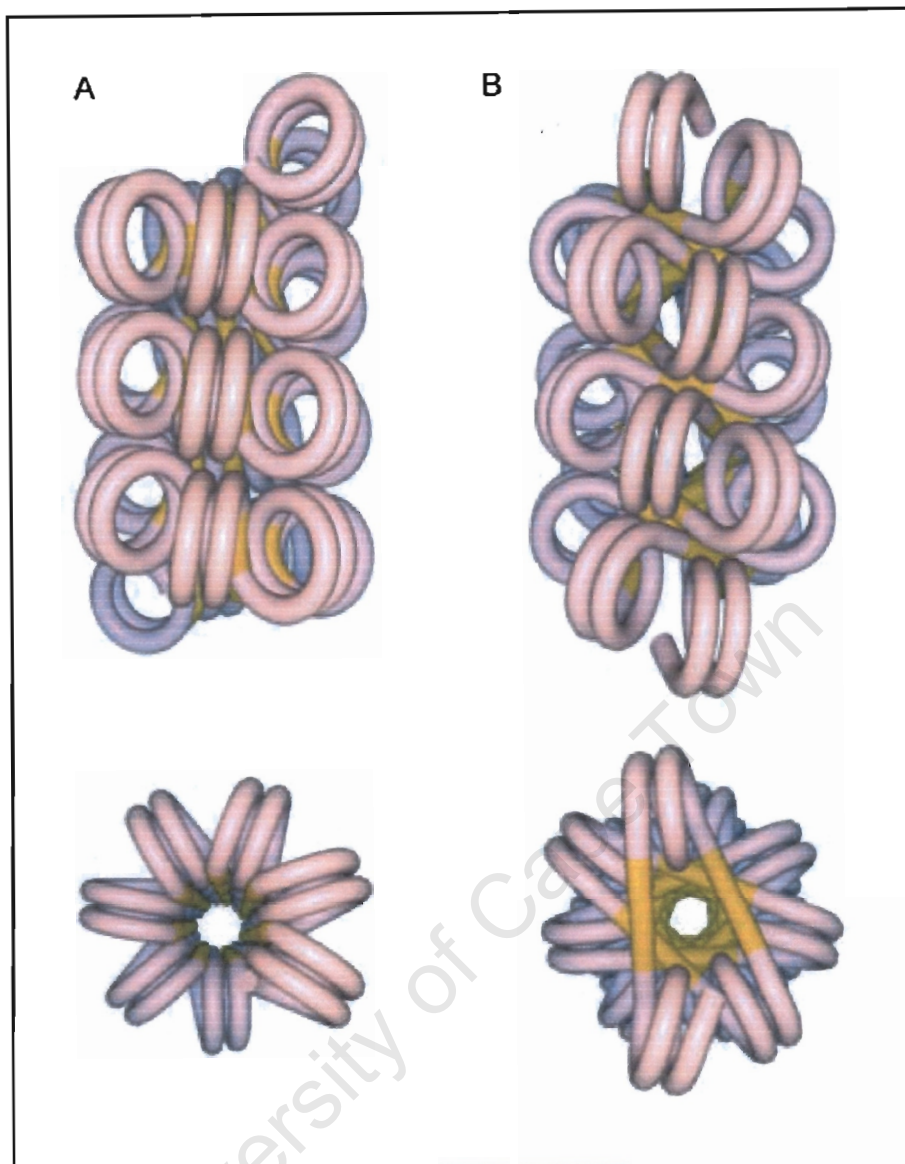


Figure 1.2. Models of one-start solenoid (A) and two-start zigzag helix (B) models. The DNA around the nucleosome core is illustrated in purple and the linker DNA in yellow. (Dorigo et al., 2004).

1.3. Nucleosome dynamics

Nucleosomes are not static structures, but are continuously unraveling and recompacting to allow DNA transcription and other essential DNA functions such as replication, recombination and repair. The most condensed state of chromatin is termed heterochromatin, whereas the relatively decondensed state is known as euchromatin. The conversion from the one to the other state is the result of the activity of many proteins, collectively termed chromatin remodeling factors.

1.3.1 Chromatin remodeling

Chromatin remodeling occurs in different parts of the genome as a result of changes in the demands of DNA function, such as the requirement for the expression of a specific gene, and has been classified into two main categories:

- a) chromatin remodeling complexes that non-covalently change the structure of chromatin, such as the ATP-dependent chromatin complexes (Vignali et al., 2000),
- b) chromatin remodeling complexes that covalently modify histones to change chromatin structure, such as by acetylation, methylation, ubiquitination and phosphorylation of the core histones (Strahl and Allis, 2000).

1.3.1.1. ATP-dependent remodeling complexes

Three mechanisms have been proposed by which ATP-dependent chromatin remodeling complexes interacted with chromatin to produce a remodeled state conducive to processes such as transcription (Flaus and Owen-Hughes, 2001):

- i. Global scanning along an array of nucleosomes by the ATP-dependent complex to find and interact with various regions of DNA and recruitment of factors without bias,

- ii. Interaction between the ATP-dependent complex and an unbound transcription activator, and subsequent recruitment of the activator-remodeler complex to the chromatin target region, and
- iii. The ATP-dependent complex interacting with an activator that is already bound to the euchromatic region of DNA.

ATP-dependent chromatin remodeling complexes all contain a highly conserved ATPase domain motif. Based on subunit composition of the chromatin remodeling complex, they have been classified into three categories:

- i. SWI/SNF complexes,
- ii. ISWI complexes, and
- iii. NURD complexes.

SWI/SNF complexes are the most extensively studied of all the ATP-dependent chromatin remodeling complexes. The yeast homologue, ySWI/ySNF, has been found to influence about 3% of the yeast genome, whereas the yeast RSC complex is involved in expression of approximately 30% of the genome (Cairns et al., 1999; Hassan, Neely, and Workman, 2001). The human homologues of SWI/SNF are BRG1 and PBAF1 (Muchardt et al., 1995; Lemon et al., 2001). In addition to the ATPase domains present in these proteins, they also contain bromodomains, which interact with acetylated lysine residues on core histone tails, as well as AT-hook domain motifs, implicated in DNA binding.

The ISWI complexes contain an ATPase domain where ATP hydrolysis is nucleosome dependant. The most studied of the ISWI complexes are ACF and CHRAC, which are involved in nucleosome assembly (Eberharder et al., 2001).

The third type of ATP chromatin remodeling complex is NURD. NURD complexes contain a Mi-2 ATPase motif. This motif consists of two PHD zinc-fingers, two chromodomains, a SWI2/SWF2 Helicase/ATPase domain and an HMG-like domain (Zhang et al., 1998; Zhang et al., 1999). Two other important

subunits are the HDAC1 and HDAC2 and MBD3 subunits. The HDAC1/2 complexes are histone deacetylases, while the MBD3 binds methylated DNA. A homologue of MBD3 is MBD2, which is part of the MeCP1 complex. This complex was shown to be composed of subunits which included the subunits of NURD (Zhang et al., 1999), suggesting that NURD complex could act by being recruited to methylated DNA by MBD2/3, such that the HDAC complexes could act on it.

This connection between the ATP-dependent remodelers and the covalent histone modification enzymes, suggests that ATP-dependent remodeling complexes could interact with post-translational modifications, and that the functions of the two classes of remodelers are intertwined, as opposed to being totally separate activities.

1.3.1.2. Post-translational Modifications

Post-translational modifications occur on the histone tails and include acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, glycosylation, biotinylation, and carbonylation (Margueron, Trojer, and Reinberg, 2005). The first three modifications are the most studied.

Acetylation occurs on lysine residues on the N-terminal tails of histones which is catalyzed by histone acetyltransferases (HATs), and removal of acetyl groups are performed by histone deacetyltransferases (HDACs). It has been postulated that the decondensation of chromatin is a result of ATP chromatin remodeling complexes, such as SWI/SNF and the acetyltransferase containing complex, SAGA. Research performed on the HO promoter in *S. cerevisiae* suggest that that SWI/SNF recruits SAGA to acetylate histones H3 and H4, thereby promoting transcription (Krebs et al., 1999). It was believed that acetylation of the histones weakened the histone-DNA interactions, resulting in DNA becoming available to transcription factors. However, recent data suggests that acetylated histones may function otherwise. When human breast cancer cells were exposed to low

doses of trichostatin A (TSA), an inhibitor of deacetylases, there was a reasonable increase in the level of acetylation of the core histones, activating the MMTV promoter, as well as promoting chromatin remodeling (Bartsch et al., 1996). In contrast, Archer and colleagues demonstrated that with high doses of TSA the level of acetylated histones did not change significantly, but that there was an inhibition of transcription (Wilson et al., 2002).

Lysine and arginine residues of N-terminal tails of histones are methylated by methyltransferases (MTase). Methylation of the two amino acid residues lysine and arginine, occur quite differently. Lysine methyltransferases contain a catalytic motif known as the SET domain. However, the MTase is not functional without the sequences that flank the SET domain, referred to as the pre-SET and post-SET domains, respectively (Rea et al., 2000). The SUV39 protein in *Drosophila* was the first isolated SET MTase that acted on lysine 9 on histone H3 (Rea et al., 2000), and was originally identified as a transcriptional repressor (Tschiersch et al., 1994). Since then, several homologues in other organisms have been identified (Bannister et al., 2001; Briggs et al., 2001; Nakayama et al., 2001), as well as other lysine residues that become methylated in histone H3 (K4 and K27) and in H4 (K20). The binding of the heterochromatin protein HP1 to K9 methylated H3 nucleosomes leads to heterochromatic silencing.

Lysine residues can be mono-methylated, di-methylated or tri-methylated. Arginine residues, on the other hand, can only be mono-methylated and dimethylated. Also, methylated arginine promotes transcription as opposed to lysine methylation. Arginine methyltransferases are more diverse in respect to their catalytic domains. The PRMT family consists of five variants, each with an affinity for arginine residues in different positions of for different histones (Kouzarides, 2002). Until recently, it was not understood how methyl groups were removed from methylated residues, as no de-methylase had been characterized. Wang and colleagues showed that human PAD4, a peptidylarginine deaminase, was capable of converting methylated arginine into

citrulline, with methylamine as the byproduct, by a process which has been termed demethyliminination (Wang et al. 2004b).

Histone kinases phosphorylate serine, threonine and tyrosine residues of N-terminal tails of histones. Recent studies into histone phosphorylation have found that it is involved in DNA damage and repair, as well as in stages in the cell cycle and was also involved in apoptosis (Ahn et al. 2005; Cheung et al., 2005). Work performed in meiotic and mitotic condensation during prophase, found that some of the core histones are phosphorylated. Phosphorylation of histone H3 serine 10 was shown to be multi-functional. During the mitotic cycle, phosphorylated H3 at serine 10, seems to participate in the formation of midbody and could play a role in cytokinesis (Li et al., 2005). In JB6 cells, phosphorylated H3 ser10 appeared to play a regulatory role in EGF-stimulated neoplastic cell transformation, providing a possible role in cancer (Choi et al., 2005). When the Ras-MAPK pathway is stimulated, H3 gets phosphorylated at serines 10 and 28, and is present in the ras-transformed cells, instead of heterochromatic regions. Also, the phosphorylation sites were found to be independent of each other, meaning that they functioned separately in the regulation of gene expression (Dunn and Davie, 2005).

A significant amount of research has been performed on H2A and its variant H2A.X, and the role of these two histones in DNA damage and repair. Immediately after a double strand break occurs, histone H2A is phosphorylated at ser 129. An SQ motif has been identified across higher eukaryotic organisms, to which a kinase, possibly a member of the PIKK family due to their appearance in response to double strand breaks, acted on (Foster and Downs, 2005). Specialized roles for ser 122 and 129 of histone H2A have been determined. Serine 122 is involved in surviving DNA damage and may have a role in homologous recombination, whereas ser 129 plays a role in responding to double strand breaks (Harvey, Jackson, and Downs, 2005).

1.4. Covalent modifications of linker histones

The linker histone H1 family has long been believed to be involved in transcriptional regulation and was thought to be a general inhibitor of transcription. Using GFP-tagged histone H1 variants in Balb/c fibroblasts to measure the dynamic binding of the protein to chromatin, it has been shown that although most of the H1 protein was bound to chromatin, it shuffled between euchromatic and heterochromatic regions (Misteli et al., 2000).

1.4.1. Linker histone phosphorylation

It was thought that phosphorylation of the linker histones were involved in chromatin decondensation, and various mechanistic models have been proposed (Roth and Allis, 1992). Cyclin dependent kinase (CDK) was shown to phosphorylate linker histones in *Tetrahymena* during the cell cycle (Roth et al., 1991). This kinase was orthologous to *cdc28p* in *S. cerevisiae*, *cdc2p* in *Schizosaccharomyces pombe* and CDK1 and CDK2 in vertebrates (Roth and Allis, 1992). Phosphorylation on the tails of the linker histone increased as the cell cycle progressed, with a maximum level detected at the G2-M stage during which mitosis occurs (Bradbury, 1992).

Two models by which linker histone phosphorylation promote transcription have been proposed. In the first model, phosphorylation of linker histones causes H1-H1 interactions via the globular domains, resulting in chromatin decondensation. Bradbury and colleagues demonstrated on the macroplasm of *Physarum polycephalum*, that there was a change in the level of H1 phosphorylation during the cell cycle. Together, with the knowledge that H1 stabilizes the supercoiling of the nucleosomes, led to the proposal of the above hypothesis (Bradbury, Inglis, and Matthews, 1974). In the second model, phosphorylation of linker histones disrupts histone tail-DNA interactions, resulting in a decondensed state (Roth and Allis, 1992; Contreras et al., 2003).

Contreras and co-workers studied the effect of CDK2 kinase on the mobility of histone H1 in immortalized mammalian cell lines (Contreras et al., 2003). The H1b isotype was studied, because it displayed the highest level of phosphorylation among the linker histone variants. A mutant, which was deficient in serine and threonine residues, was generated and fused to the green fluorescent protein (GFP). The wild type H1b was also labeled with a GFP tag. Using fluorescence recovery after photobleaching (FRAP) methodology, it was found that the mutated H1b had a lower mobility than the wild type. Also, it was shown that CDK2 activity was associated with H1, and when CDK2 was inhibited by p21, there was a decrease in the mobility of the GFP-H1b, but not the mutant fusion protein. When comparing the exchange dynamics of a cyan fluorescent protein-tagged H1 and its mutant in heterochromatin *versus* euchromatin, the shuffling of both proteins were similar in euchromatin, dissociation of the mutant protein was decreased in heterochromatin. The authors concluded that H1 was phosphorylated by CDK2, and that this post-translational modification influenced its mobility. Also, H1 was dephosphorylated in heterochromatic regions (Contreras et al., 2003), providing evidence that phosphorylated H1 was involved in chromatin decondensation, and possibly transcription.

It was demonstrated in *Tetrahymena* that a mutant H1 that mimicked the constitutive phosphorylated state did not control gene expression globally, but rather that genes were regulated as in the absence of H1. The *ngoA* gene was found to be activated and the *CyP1a* gene expression reduced (Dou et al., 1999). Further work to elucidate the process by which the phosphorylated H1 regulated these genes were performed, by studying the effect of a mutant mimicking phosphorylated H1, as well as other mutants that placed a negatively charged patch at different locations in the H1 molecule. These experiments showed the repression of the *CyP1* promoter, whereas dephosphorylated H1 allowed *CyP1* to function normally in starved *Tetrahymena* cells. This suggested that the

phosphorylation of H1 generated a charged patch, which functioned to regulate the transcription of a select set of genes (Dou and Gorovsky, 2000).

Gorovsky and co-workers were also interested in finding additional genes that were regulated by histone H1 phosphorylation in response to starvation, and used subtractive hybridization to identify such genes. They found a set of genes which included the CYP1a gene, encoding a cysteine protease, that were upregulated during starvation due to the dephosphorylation of H1. When okadaic acid was used to inhibit phosphatases, which left H1 phosphorylated, the CDC2 gene was found to be up regulated, showing a positive feedback loop between phosphorylated H1 and CDC2 expression. The authors propose that when cells were starved, H1 was dephosphorylated by a phosphatase, and protease genes were expressed, leading to the degradation of proteins to provide amino acids essential to survival of the *Tetrahymena* cell (Dou et al., 2005).

Additional evidence for a role for phosphorylated H1 in the regulation of transcription comes from work on the MMTV promoter by the Archer lab. Glucocorticoid hormone stimulated chromatin remodeling on the promoter, which resulted in an initial increase in transcription. Prolonged exposure, however, inhibited transcription, and caused dephosphorylation of H1. Removal of glucocorticoid resulted in the rephosphorylation of H1, as well as the reinitiation of transcription from the MMTV promoter (Lee and Archer, 1998). Studies were also performed to determine if cdk2 kinase was implicated in the dephosphorylation of H1 due to overlong exposure of the cells to glucocorticoid. *In vivo* and *in vitro* studies confirmed that cdk2 activity was reduced in glucocorticoid treated cells, and an upregulation of the cdk2 inhibitor, p21, was seen. The fact that cdk2 activity and phosphorylation of H1 correlated, led the authors to conclude that cdk2 was indeed the kinase involved (Bhattacharjee et al., 2001).

1.4.2. H1 and core histone acetylation

In vitro studies performed on dinucleosome templates that contained purified core histones showed that histone octamers displayed a mobility which was a function of the DNA sequence, and reduced transcription compared to naked DNA. Addition of linker histone H5, an extreme variant of H1, resulted in the repression of transcription and restricted nucleosome mobility (Ura, Hayes, and Wolffe, 1995). These authors further demonstrated both *in vitro* and *in vivo* in *Xenopus* egg extracts, that acetylated core histones promoted transcription significantly, but when linker histone was added, transcription and nucleosome mobility was repressed (Ura et al. 1997).

Perry and co-workers found that treatment of cells with sodium butyrate, an inhibitor of deacetylases, resulting in highly acetylated core histones, produced chromatin that was generally a less condensed, as well as deficient of H1 (Perry and Annunziato, 1991). Additional evidence for a possible link between the modification state of the core histones, and the ability of the linker histone to bind to chromatin came from *in vitro* studies on chicken erythrocytes, which demonstrated that acetylated core histones reduced the binding of H1, and restrained the formation of a compact chromatin (Ridsdale et al., 1990).

Work on retinoic acid receptors (RARs) have demonstrated that the RARs interact with HMG-I, a high mobility group protein which is known to bind H1, and which could be an early step in the decondensation of chromatin. The absence of H1 leads to the recruitment of histone acetyltransferase (HAT) complexes by transcription factors, which, in turn, acetylates the core histones, thereby destabilizing the stable, condensed chromatin structure (Nagpal et al., 1999). Studies were performed on oligonucleosome arrays where it was shown that the HAT, PCAF, could not acetylate core histones in the presence of H1, but could easily acetylate free core histones, and, in addition, the inhibition of acetylation was determined to be a result of steric hindrance of the H3 tail by H1 (Bustin,

Catez, and Lim, 2005;Herrera, Schiltz, and Bustin, 2000). Excessive amounts of the H1 variants, H1c and H1^o, were shown to reduce the level of core histone acetylation in mammalian cell lines (Gunjan, Sittman, and Brown, 2001). Hyperacetylation of core histones resulted in the rapid dissociation of chromatin-associated GFP-H1 (Herrera, Schiltz, and Bustin, 2000;Misteli et al., 2000). Based on these findings, H1 needs to be removed or remodeled, possibly by phosphorylation or binding to a HMG protein, to allows core histone acetylation, and efficient gene activation.

1.4.3. DNA Methylation and H1

Methylation occurs on CpG islands in DNA, and is linked to chromatin condensation. A series of studies, sedimentation assays, gel retardation assays and southwestern analyses, showed that H1 preferentially bound to methylated DNA as opposed to unmodified DNA (McArthur and Thomas, 1996). Contradictory evidence was found in other studies, where DNA methylation had no influence on H1 binding (Campoy et al., 1995). Strom suggested that hypomethylated linker DNA influenced H1-linked chromatin condensation (Caiafa et al., 1995). H1 was also shown to inhibited methylation of AT-rich as opposed to GC-rich DNA (Carotti et al., 1996), although this may have been related to a sequence specificity of H1 binding (Wellman, Song, and Mamoon, 1999)

Further evidence for a link between H1 binding and the methylation state of the DNA came from Zlatanova, who showed, by atomic force microscopic studies (AFM), that DNA methylation was a pre-requisite for H1 binding to reconstitute the chromatin fiber (Karymov et al., 2001). Van Holde addressed the contradictory findings concerning DNA methylation and H1 binding, pointing out that the various research groups used different H1 variants from different organisms, isolated under different conditions and cell cycle stages (Zlatanova, Caiafa, and van Holde, 2000). MeCP2 is a protein that occurs abundantly in mammalian cells, and was found to associate with methylated DNA and repress

transcription. This is of interest because MeCP2 is capable of displacing H1 to gain access to methylated DNA (Nan, Campoy, and Bird, 1997).

1.5. The Histone Code hypothesis

The histone code hypothesis was independently proposed by Turner and by Allis, and have been extensively reviewed (Berger, 2002; Wang et al., 2004a; Iizuka and Smith, 2003; Cosgrove, Boeke, and Wolberger, 2004; Margueron, Trojer, and Reinberg, 2005). It was suggested that covalent modifications to core histones act in concert and/or with other proteins to regulate DNA function, most likely by modifying chromatin structure and recruit specialized non-histone proteins to specific locations in the genome (Strahl and Allis, 2000).

The criteria that would need to be met:

- a) Modifications at specific residues on histone tails would lead to the recruitment of proteins that associate with chromatin,
- b) Various combinations of modifications can be generated on the same or different histone tail on any particular nucleosome,
- c) The behavior of heterochromatic and euchromatic regions are dependent on the modifications made to histone tails of the nucleosomes (Jenuwein and Allis, 2001).

1.6. Project Aim

The phosphorylation level of linker histones have been shown to change under varying physiological conditions (Roth et al., 1988), and a link between dephosphorylated H1 and chromatin condensation was established (Roth and Allis, 1992). The biochemical mimicking of the phosphorylated state of H1 in *Tetrahymena* caused an increase in the dynamic exchange of H1 on chromatin (Dou et al., 2002). Archer and colleagues also demonstrated that prolonged

treatment of mouse cells with dexamethasone resulted in the dephosphorylation of three histone H1 variants, as well as an inhibition of transcription of the MMTV promoter (Banks et al., 2001). A further regulatory role was proposed when Peterson and co-workers showed that SWI/SNF remodeling is inhibited by the incorporation of linker histones into nucleosomal arrays, but is rescued by the Cdc2/Cyclin B kinase phosphorylation of the linker histones (Horn et al., 2002). Based on these findings, the phosphorylation of H1 was proposed to decrease the affinity of H1 for chromatin, resulting in H1 dissociation which, in turn, allowed the recruitment of ATP-dependent remodeling complexes and core histone tail modification enzymes, ultimately resulting in the decondensation of chromatin and unmasking of the DNA molecule.

We set out to determine whether yeast Hho1p was phosphorylated or dephosphorylated at any stage. It was previously shown in our group that Hho1p displayed a significant increase in binding to chromatin in stationary phase, and that this was accompanied by an increase in chromatin compaction (Schafer et al., manuscript in preparation). We were interested in assessing whether this change in the binding of Hho1p to chromatin was regulated by phosphorylation of the Hho1p protein during entry and exit from stationary phase.

Chapter 2

Materials and Methods

2.1 Yeast strains

The *Saccharomyces cerevisiae* strain *JDY43*, a kind gift from Jessica Downs (Downs et al., 2003), was used for the isolation of Hho1p. *JDY43* expresses a single Hho1p from the native *HHO1* promoter, and contains twelve repeats of a C-terminal c-myc tag.

2.2 Determination of the phosphorylation states of Hho1p

2.2.1 Growth conditions

Liquid YPD medium (500ml) was inoculated with a single colony of *JDY43* and incubated overnight at 30°C with orbital shaking (200rpm). The following day 100ml of the starter culture was transferred to 400ml pre-warmed liquid YPD medium, and returned to 30°C with agitation. Aliquots (50ml) were withdrawn from the culture 4h, 1d, 2d, and 6d after inoculation. On the sixth day, 100ml of the culture was used to inoculate 400ml of fresh pre-warmed liquid YPD, incubated at 30°C with agitation. Sample volumes of 50ml was withdrawn from this culture 1h, 2h and 4h after inoculation.

2.2.2 Protein extraction

S. cerevisiae cultures were centrifuged in a Beckman SX4750A swing-out rotor at 2800rpm at 4°C, and the pellets resuspended in 1ml ice-cold 1× phosphate buffered saline (PBS). Cells were disrupted by vigorous vortexing (Scientific Industries Vortex Genie 2) in the presence of 100µl of glass beads (Sigma) at 4°C for 1h. Cells were pelleted at 10000rpm at 4°C in a microfuge (Eppendorf) and the supernatant collected.

2.2.3 Protein concentration determination

Protein concentrations determined with a Bradford Assay kit (Bio-Rad). All absorbances were recorded at 595nm, and concentrations calculated using a bovine serum albumin (BSA) standard series.

2.2.4 Protein Immunoprecipitation

The above-mentioned experiment was adapted from Current Protocols (Ausubel et al., 1997) with the alterations to the scale of the experiment.

2.2.4.1 Preparing the Antibody bound Sepharose

An aliquot (20µl) of 50% Protein-A-Sepharose CL-4B (Amersham Biosciences) was pre-incubated with 4µl anti-c-myc antibody (0.4µg/µl, Sigma) and 500µl 1× PBS in a microfuge tube at 4°C overnight on a rolling cylinder shaker. The antibody-bound Sepharose CL-4B was centrifuged at 10000rpm in a microfuge, and the aqueous layer removed. The antibody-bound Sepharose beads were then washed three consecutive times with 500µl non-denaturing lysis buffer (1% (v/v) Triton X-100, 50mM Tris-Cl pH 7.4, 300mM NaCl, 5mM EDTA, 0.02% (w/v) sodium azide, 1× protease inhibitor cocktail (Roche)).

2.2.4.2 Binding the protein to the antibody-bound Sepharose

Protein sample (6µg) was dispensed into microfuge tubes containing antibody-bound Sepharose and 10µg BSA to reduce non-specific association. The volumes in each tube was adjusted to 500µl with 1× PBS, and the microfuge tubes were then placed at 4°C on the rolling cylinder shaker for 2h. The samples were spun down (10000rpm, 10s) and the supernatant was removed. The antibody-bound Sepharose was washed with 500µl wash buffer (0.1% (v/v) Triton X-100, 50mM Tris-Cl pH 7.4, 300mM NaCl, 5mM EDTA, 0.02% (w/v) sodium

azide, 1× protease inhibitor cocktail) three times at 4°C. Finally, most of the 1× PBS was removed leaving behind approximately 20µl.

2.2.4.3 SDS-PAGE gel electrophoresis

SDS-PAGE gels were prepared using established protocols. The separating gel consisted of 6% (w/v) acrylamide:bisacrylamide 29:1 (Bio-rad), 380 mM Tris-Cl pH 8.8 and 0.1% (w/v) sodium dodecylsulphate (SDS). The stacking gel consisted of 5.6% (w/v) acrylamide:bisacrylamide 29:1 (Bio-rad), 125mM Tris-Cl pH 6.8 and 0.1% (w/v) SDS. Sample application buffer (6×: 15% (v/v) glycerol, 0.125mM Tris-Cl pH 6.8, 5 mM EDTA, 2% (w/v) SDS, 0.1% bromophenol blue) was added to all protein samples. Samples were adjusted to 1% β - mercaptoethanol (1%), heated at 95°C, loaded onto the SDS-PAGE gels and electrophoresed in the presence of 1× electrophoresis buffer at 50V for 2 hours in a MiniProtean III (Biorad) electrophoresis system.

2.2.2.4 Western Blot

After the electrophoretic separation of proteins, the gel was re-assembled in the western blotting apparatus (Bio-Rad), where it was placed against a positively charged nitrocellulose membrane (Amersham Biosciences), submerged in transfer buffer (10% (v/v) methanol, 1× electrophoresis buffer), and the protein transferred overnight at 15V at 4°C.

Following the protein transfer the membrane was blocked with fat-free milk powder (5% (w/v) milk powder in 1× PBS/Tween (80mM disodium hydrogenphosphate, 20mM sodium dihydrogenphosphate, 100mM NaCl, 0.1% (v/v) Tween 20)) in the case of the anti c-myc antibody. When blocking for the phosphoserine antibody, the membrane was blocked with 3% (w/v) BSA in 1× PBS/Tween. For the phosphothreonine antibody, the membrane was blocked

with 5% (w/v) BSA (dissolved in TBS buffer (20mM TRIS-Cl, 500mM NaCl, 0.05 % (v/v) Tween 20, 0.2 % (v/v) Triton X-100, pH 7.5) in 0.1% (v/v) Tween 20).

The membrane was rinsed in 1× PBS/Tween (TBS buffer for phosphothreonine antibody) and incubated with the primary antibody diluted in 1× PBS/Tween. In the case of the anti c-myc antibody (0.4µg/µl, Roche) the preparation was diluted 1:10000, in the case of the anti-phosphoserine (0.25µg/µl, Abcam), 1:1600, and in the case of the anti-phosphothreonine (0.5µg/µl, Abcam), 1:200. All incubations were performed overnight at 4°C on the rolling cylinder shaker. Following three consecutive washes in 1× PBS/Tween (TBS buffer for phosphothreonine antibody) for 10 minutes each, the appropriate secondary antibody at required dilution were added. Anti-mouse antibody (1:10 000, Amersham Biosciences) was used for the anti-c-myc antibody and anti-rabbit antibody for the anti-phosphoserine and anti-phosphothreonine antibody (1:10 000, Amersham Biosciences). The nitrocellulose membrane was washed three times to remove excess antibodies, and the protein bands were visualized using enhanced chemiluminescence (ECL kit, Amersham Biosciences) as directed by the manufacturer.

2.2.4.1 Stripping the membrane

The membrane was incubated with 20ml of stripping buffer (2% (w/v) SDS, 600mM TRIS-Cl pH 8.8) containing 175µl β-mercaptoethanol for a period of 45 minutes at 57°C in a rotating hybridisation oven (Hybaid). The membrane was rinsed thoroughly with 1× PBS/Tween to remove the β-mercaptoethanol.

2.3 Immuno slot/dot blotting

Protein samples were loaded into the wells of the immuno blotting apparatus, and transferred to the nitrocellulose membrane by water vacuum suction. The

membrane was subsequently blocked, probed and developed as described above.

2.4 Affinity chromatography

2.4.1 Protein extraction for affinity chromatography

Cultures of *JDY43* (4l) were grown for six days at 30°C. The cultures were centrifuged at 10000rpm at 4°C in a JA-14 rotor and the pellet washed twice in 200ml water. The pellet was resuspended in 50ml 1× PBS containing 2mM PMSF. A crude protein extract was prepared by adding glass beads (Sigma) and vigorous shaking on a vortexer at 4°C for 2 hours. The cellular debris and glass beads were pelleted by centrifugation at 2800rpm at 4°C in a Beckman Allegra 6R benchtop centrifuge.

2.4.2 Preparing the column

The c-myc tagged histone Hho1p was purified by affinity chromatography using an anti-c-myc agarose conjugate (Sigma-Aldrich). The column was packed and the protein eluted according to the manufacturer's instructions. The 1ml resin was packed into a 25ml column, washed three times with 5ml volumes of 100mM ammonium hydroxide (Sigma Aldrich), followed by three 5ml volumes of 1× PBS.

2.4.3 Binding and eluting the protein

The protein extract was loaded onto the column under gravity flow and the flow through collected. The column was then washed with 1× PBS until the absorbance readings at 280nm were less than 0.01 units. Histone Hho1p-c-myc was eluted from the column with 10ml 100mM ammonium hydroxide. Fractions were collected into microfuge tubes that contained 120µl 1M acetic acid.

2.5. Perchloric acid extraction of Histone Hho1p

This protocol was based on a perchloric acid (PCA) extraction method described by Albig and co-workers (Albig et al., 1998). A 4l culture of JDY43 was grown in liquid YPD overnight, and a second culture for six days. The cells were pelleted at 2900rpm in a JA-20 rotor for 10 minutes at 4 °C, and washed in 30ml water. The pellet was resuspended in 20ml PTB buffer (1M sorbitol, 4.25mM KH_2PO_4 , 45.75M K_2HPO_4) and 20mM dithiothreitol (DTT), and incubated at 37 °C for 30 minutes. Following incubation, the suspension was pelleted at 5000rpm for 5 minutes at 4°C, and resuspended in 15ml DB buffer (1M Sorbitol, 1mM EDTA) and 10mM zymolyase T20 (Seigegaku) and returned to 37 °C for 30 minutes. The resuspended pellet was centrifuged at 5000rpm for 10 minutes at 4 °C. The pellet was washed with DB buffer. A pellet volume of glass beads (Sigma) and 1ml 5% PCA was added to the samples and vortexed for 2 hours and then incubated on ice for 30 minutes. The samples were then precipitated with 20 % trichloroacetic acid (TCA) and centrifuged at 12000 rpm for 10 minutes at 4 °C. The pellet was solubilized in 10mM hydrochloric acid, with brief incubations on ice between stirring.

Where further purification was required, the histone preparation was saturated with 90% saturated $(\text{NH}_2)\text{SO}_4$, incubated on ice for 1h, and then pelleted at 12000 rpm for 10 minutes. The pellet was again solubilized in 10mM HCl. A TCA precipitation was performed to remove all traces of $(\text{NH}_2)\text{SO}_4$ and a finally resolubilized in 10mM HCl.

2.6 Sephadex CM52 purification of histones

Briefly, histone Hho1p samples prepared by perchloric acid extraction were applied to a 1ml Sephadex CM52 cation exchange resin that had been equilibrated with phosphate buffer (10mM sodium phosphate pH 7.0, 0.25mM EDTA, 50mM NaCl, 0.25mM PMSF). Fractions of 500µl were collected and analysed by SDS-PAGE.

2.7 MALDI-TOF mass spectrometry

2.7.1 Silver Staining

The gel was fixed overnight in 50% (v/v) ethanol, 10% (v/v) acetic acid, incubated with 30% (v/v) ethanol for 15min and subsequently washed three times with distilled water at 5 minute intervals. The gel was sensitized with 0.2 g/l freshly made sodium thiosulphate solution for 1.5 minutes. The gel then washed three times with distilled water for 30s each, and incubated with 2 g/l silver nitrate for 25 minutes. The silver nitrate solution was removed by washing three times with distilled water for 30s each. The gel was developed by incubating in developing solution (60g/l sodium carbonate, 20 ml 0.2g/l sodium thiosulphate solution and 500 μ l formaldehyde). When the desired level of staining was achieved, development was quenched by removing the developing solution and adding 6% (v/v) acetic acid for 10 minutes. The gel was then washed with distilled water four times at 15 minute intervals.

2.7.2 MALDI-TOF mass spectrometry

Bands were excised from the SDS-PAGE gels and sent to the MALDI-TOF facility where tryptic digests were performed .

BSA was used as a positive control and a blank gel piece was used as a negative. The gel slices were washed twice in 200 μ l 50% (v/v) acetonitrile, 25 mM NH_4HCO_3 for 15s and then washed in 200 μ l 100% (v/v) acetonitrile. After drying the gel slices for 30 minutes, each band was digested with a final concentration of 1 μ g/ml trypsin in 25 mM NH_4HCO_3 overnight at 37 °C. 50% (v/v) acetonitrile and 5% TFA was added to the samples and incubated for 30 minutes. Samples were dried and redissolved in 0.05% (v/v) TFA, 5% (v/v) acetonitrile and an equal volume of α -cyano-4-hydroxycinnamic acid (10 mg/ml in 60% (v/v) acetonitrile, 0.3% (v/v) TFA) was added. A 2 μ l aliquot was applied to a gold plated MALDI-TOF grid and inserted into a MALDI-TOF spectrophotometer

(Perspective Biosystems Voyager DE.Pro). The tryptic digests and MALDI-TOF was performed by Lara Donaldson, the MALDI-TOF technician. Proteins were identified by comparing the peptide mass sizes to databases utilizing MS-Fit (<http://prospector.ucsf.edu>).

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Chapter 3

Results

3.1. Does the phosphorylation state of Hho1p change in stationary phase?

We have shown that the *Saccharomyces cerevisiae* linker histone Hho1p exhibited a significant increase in binding to chromatin in stationary phase (Schafer et al., manuscript in preparation). Although there has been some work on the covalent modifications of linker histones in the cell cycle and during starvation in *Tetrahymena* (Roth and Allis, 1992; Bradbury, 1992; Dou et al., 2005), there has never been a clear demonstration of a significant increase in linker histone binding to chromatin in a defined physiological phase. The passage of a yeast cell to stationary phase and concomitant increase in Hho1p binding to chromatin therefore provided us with an ideal system in which to study the mechanism that regulated the association of Hho1p with chromatin.

In the work reported here, we were particularly interested in studying possible reversible covalent modifications of Hho1p in the transition to stationary phase. Because of the clear role that phosphorylation was shown to play in chromatin binding during the mitotic cycle (Roth and Allis, 1992; Bradbury, 1992) and the inferred relationship between phosphorylation and the dynamic exchange of linker histones between chromatin and the solute (Dou et al., 2002; Contreras et al., 2003), we focused our study on a possible change in the phosphorylation state of Hho1p during entry into stationary phase.

3.2. Western blots of the immunoprecipitations

Yeast Hho1p contains 33 serine, 18 threonine and 6 tyrosine residues (see Figure 3.1). There is currently no recorded information on the phosphorylation of

any residue in Hho1p (<http://phospho.elm.eu.org>). An analysis of potential phosphorylation sites in Hho1p (see Figure 3.1) identified 6 protein kinase A, 11 protein kinase C, and 2 potential CDC28 phosphorylation sites, although both the latter sites contained one mismatch to the consensus sequence. Since the preponderance of phosphorylation of serine and threonine residues is significantly higher than that of tyrosine, we decided to concentrate on the possibility of serine or threonine phosphorylation/dephosphorylation of Hho1p during entry into stationary phase as a mechanism controlling binding of Hho1p to chromatin.

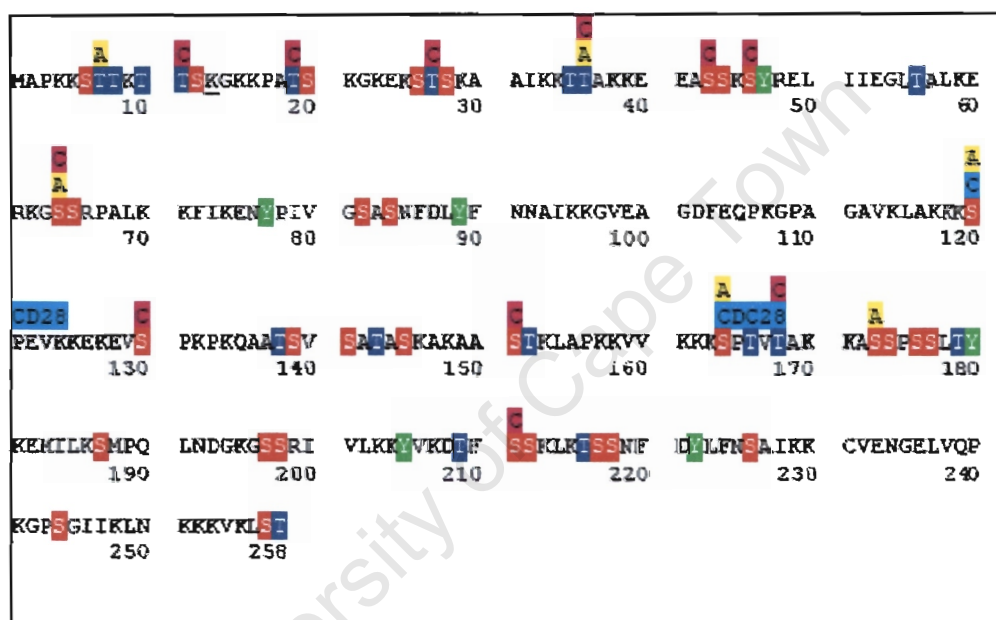


Figure 3.1. Potential phosphorylation sites in *Saccharomyces cerevisiae* histone Hho1p. Serine, threonine and tyrosine residues are shown by red, blue and green boxes, respectively. Potential sites of protein kinase A (consensus sequence R/K-R/K-X-S/T), protein kinase C (consensus sequence S/T-X-R/K) and CDC28 (consensus sequence R/K-S/T-P-X-R/K) are indicated by the yellow, purple and azure boxes, respectively. The two indicated possible CDC28 sites have one mismatch each.

The *JDY43* strain of *S. cerevisiae*, expressing a C-terminally c-myc tagged Hho1p as the only linker histone, was grown for a six-day period to stationary

phase (Werner-Washburne et al., 1993), and the cultures transferred to fresh rich media, allowing the cultures to exit stationary phase and re-enter the cell-cycle. Samples were taken at different times during entry and exit of stationary phase, the Hho1p histone immunoprecipitated with anti c-myc antibodies from total protein extracts, resolved on an SDS-PAGE gel, and western blotted with the antibodies indicated.

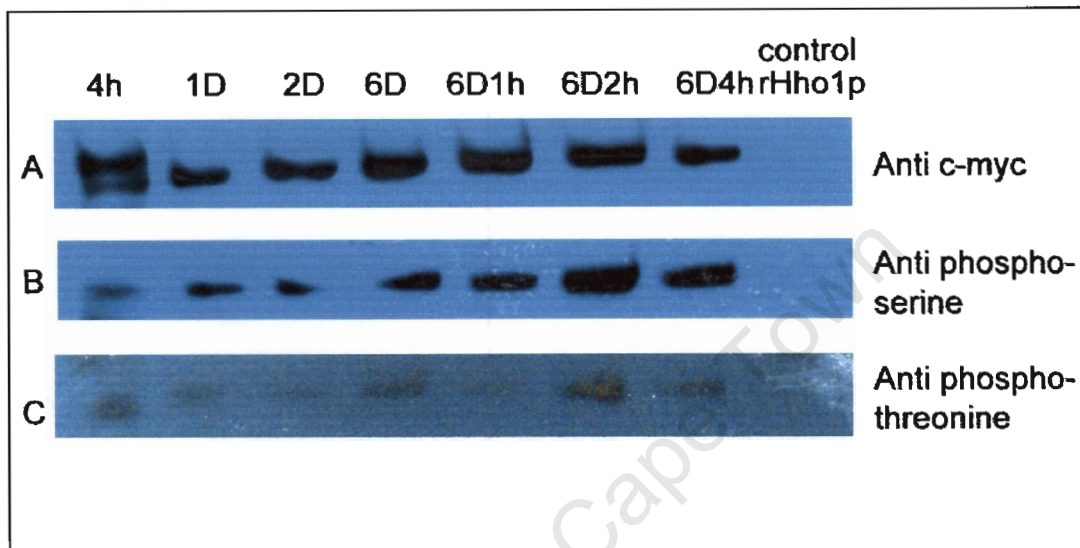


Figure 3.2. Western blots of Hho1p immunoprecipitated from *S. cerevisiae* in different growth phases. **A** Anti c - myc antibodies were used to confirm the presence of c-myc tagged Hho1p. **B** Probing with anti phosphoserine and **C**, anti phosphothreonine antibodies to investigate the presence of phosphorylated serine and threonine residues in Hho1p.

It is evident from Figure 3.2 that comparable amounts of c-myc tagged Hho1p were loaded into the different gel lanes. The filter shown in panel A was stripped, reprobed with anti-phosphoserine (Figure 3.2B) antibody, stripped, and reprobed with anti-phosphothreonine (Figure 3.2C) antibody. A visual inspection of panel B suggested that a low level of phosphorylated serine was present in Hho1p, and that this level increased during growth to stationary phase. There was no obvious trend in the phosphorylation level of threonine in the Hho1p during the experimental time course. The anti c-myc, phosphoserine and phosphothreonine

antibodies appeared to recognise the appropriate epitopes, since the antibodies did not cross-react with recombinant Hho1p lacking the c-myc tag, and which was not expected to be phosphorylated in *E. coli*. To confirm this visual impression of the phosphorylation levels, we integrated the volume of each band at the different times (Figure 3.2B and C), and normalised this volume to that of the anti c-myc probed band (Figure 3.2A) to correct for differences in IP yield and loading. This normalised phosphorylation levels are shown in Figure 3.3

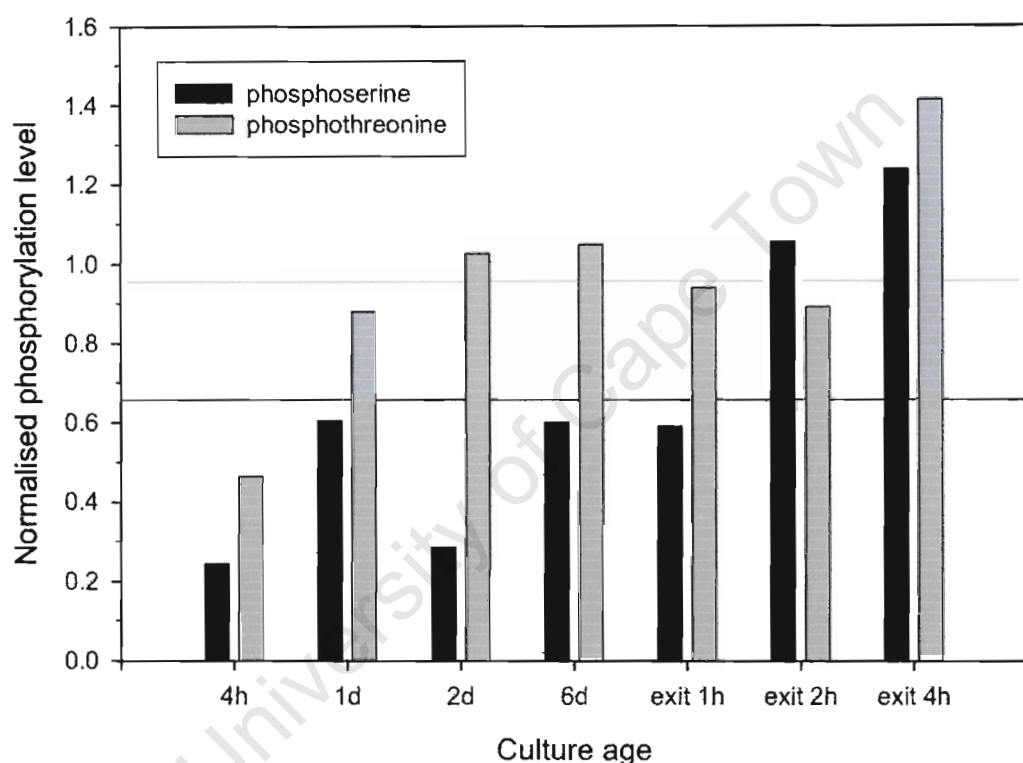


Figure 3.3. Phosphorylation levels of serine and threonine residues in Hho1p isolated from yeast at different times during entry and exit of stationary phase. The histogram shows the quantitated ratios of the band volumes for the anti phosphoserine western (Figure 3.2B) and anti phosphothreonine western (Figure 3.2C) expressed relative to that of the corresponding band volumes for the anti c-myc western (Figure 3.1A). The average normalised phosphoserine and phosphothreonine values are shown by the black and gray lines, respectively.

Referring to Figure 3.3, it is evident that the trend of serine phosphorylation remains constant during growth to stationary phase, with a slight increase during later stages in stationary phase exit. A similar trend is seen for threonine phosphorylation, although the increase 4h after exit from stationary phase was only 47% higher than the average, compared to 82% seen at the same time point for the phosphoserine value. However, an independent repetition of this experiment did not show a similar trend.

We were concerned by the possibility that one or more residues may remain phosphorylated during entry or exit from stationary phase, which was expected to cause only a small change in the detectable total phosphorylation level of the protein with the complete phosphorylation/dephosphorylation of a single residue. For instance, if only one of three residues became totally dephosphorylated during growth to stationary phase, the use of antibodies would ideally detect only a 33% reduction in the phosphorylation level. We therefore considered alternative methods to study the modification of Hho1p during passage to stationary phase, as detailed below.

3.3. Hho1p extraction

In order to confirm our findings using antibodies directed against phosphoserine and phosphothreonine, we aimed to immunoprecipitate Hho1p at different times during growth to and exit from stationary phase, purify the linker histone, and perform matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric analysis on the purified protein. However, we could never produce sufficient yields of immunoprecipitated Hho1p suitable for MALDI-TOF analysis. We therefore proceeded to scale up the isolation procedure, by making use of affinity chromatography.

3.3.1. Protein purification via the c-myc column

Total protein was isolated from one and from six day cultures of *JDY43*, expressing only a c-myc tagged Hho1p as linker histone, and the protein preparation passed through a c-myc affinity column. Elution fractions were collected, aliquots applied to a membrane, and the membrane probed with c-myc antibody to determine the chromatographic distribution of Hho1p (Figure 3.4). Fractions from both the 1 day and 6 day contained the c-myc tagged Hho1p (1 day: rows A and C; 6 day: rows E and G). To demonstrate the efficiency of the column and that all Hho1p-c-myc protein was eluted, the flow through of all the fractions were also applied to the membrane (1 day: rows B and D; 6 day: rows F and G). These fractions did not contain any sign of the tagged protein except with a little showing up at the 6 day (row 8). This experiment showed that we could purify the c-myc tagged Hho1p protein from one and six day cultures by anti c-myc affinity chromatography.

3.4. Silver stain of the samples prior to MALDI-TOF

In order to establish the purity of the c-myc affinity preparation, fractions from the c-myc affinity eluate that contained Hho1p were pooled, concentrated, and resolved on a SDS-PAGE gel (Figure 3.5). The c-myc tagged Hho1p, which contained twelve repeats of the c-myc sequence, was previously shown to resolve at approximately 65kDa on a SDS-PAGE gel (Downs et.al, 2003). Referring to the gel (Figure 3.5), it is clear that both the one day and the six day affinity purified fractions contained numerous contaminating proteins, and that an additional purification step had to be undertaken to provide sample of a homogeneity suitable for MALDI-TOF mass spectrometric analysis. Unfortunately, the protein samples were not pure and contained many other proteins. We therefore decided to excise four gel slices containing the major protein bands in the 50 to 70kDa range for the further MALDI-TOF analysis.

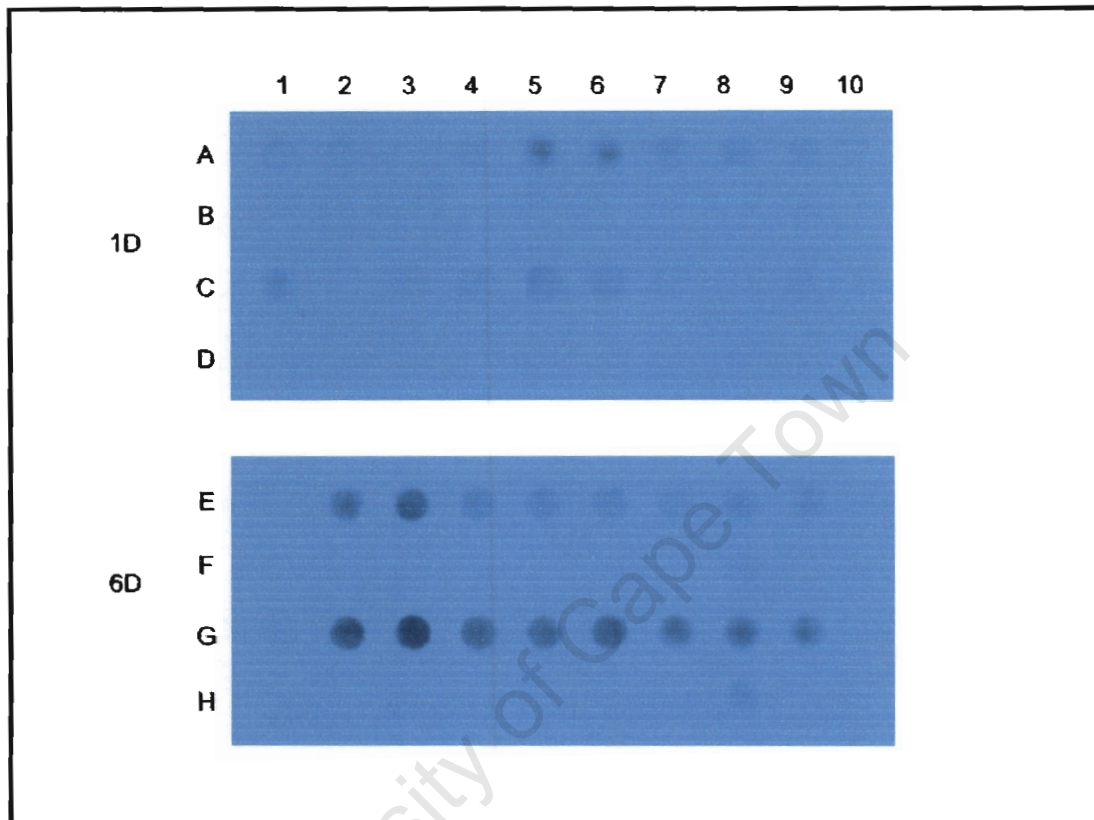


Figure 3.4. Western blots of the 1 day (1D) and 6 day (6D) fractions eluted from a c-myc affinity column. Rows A and C show duplicates of the 1D fractions across lanes 1 to 10 while rows B and D were the flow through of the column. Rows E and G were 6D fractions and rows F and H were the flow through.

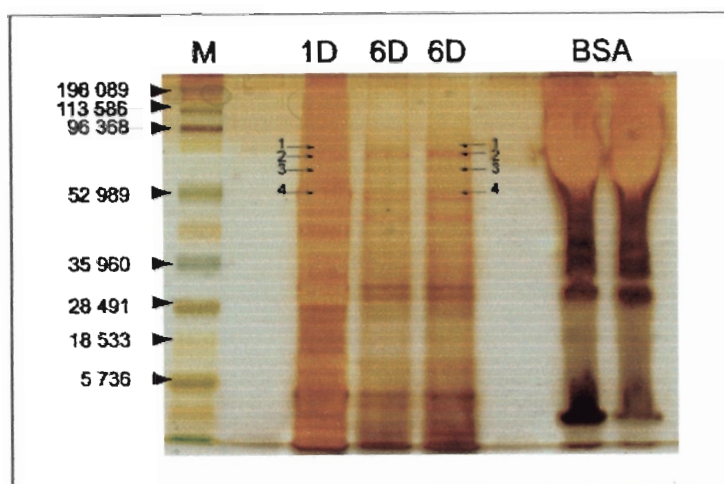


Figure 3.5. Silver stain of one day (1D) and six day (6D) samples purified by affinity chromatography. A broad range marker (lane 1) was used to determine the sizes of the bands. Lanes 3-5 show 20 μ l fractions of 1D and 6D and lanes 7-8 contained 5 μ l 3%(w/v) bovine serum albumin which were controls for the silver stain. The four major protein bands that were excised for further analysis are indicated.

3.5. MALDI-TOF mass spectrometry

The MALDI-TOF mass spectra were analysed and potential protein identities determined with MS-Fit software (<http://prospector.ucsf.edu>). The default configuration on MS-Fit required four matched peptide m/z ratios and one missed cleavage for identification of the protein against the yeast protein database. Since the samples were likely to contain contaminating proteins, we expected that only a few of the recovered peaks in each spectra might match that expected of Hho1p. The contribution of the c-myc dodecamer peptide was also considered in the analysis.

3.6. Identification of the protein in band 1

The mass spectra of a partial tryptic digest of the proteins in band 1 (see Figure 3.5) is shown in Figure 3.6. There were clear similarities between the spectra recorded for the protein samples isolated from the one day and from the six day cultures. The positions of major peaks present in the samples from both cultures are shown by arrowheads (Figure 3.6). We also noted subtle differences, particularly in the central regions of the recorded spectra. This could be due to real differences in the protein identities and levels of the two cultures. It was previously shown that the protein composition of *S. cerevisiae* cells in stationary phase differed from that of exponentially growing cells (Werner-Washburne et al., 1993). Alternatively, such differences could also be due to protein hydrolysis or variation in the extent of tryptic digestion of the two samples, resulting in differences in the mass distribution of generated peptide fragments.

The identification of a unique protein, using only the mass spectrometry spectra, proved difficult. Using the default settings of the MS-Fit program, several imperfect matches to the theoretical spectra of yeast proteins were obtained, including kinase-like proteins and a glutamyl-tRNA reductase. The poor match between the experimental and theoretical spectra was most likely due to the presence of a complex mixture of proteins in the excised gel slice. In an attempt to try and identify a unique protein, specifically the c-myc tagged Hho1p, the search was repeated, but with a relaxed stringency settings. This analysis identified oxidases, ribosomal proteins and DNA repair proteins. The most likely result from the dataset was the *S. cerevisiae* RNA polymerase-associated protein 1 (Paf1p). This result suggested that Hho1p did not resolve in slice 1 recovered from the SDS-PAGE gel, and we continued the analysis of the proteins in the other recovered gel slices.

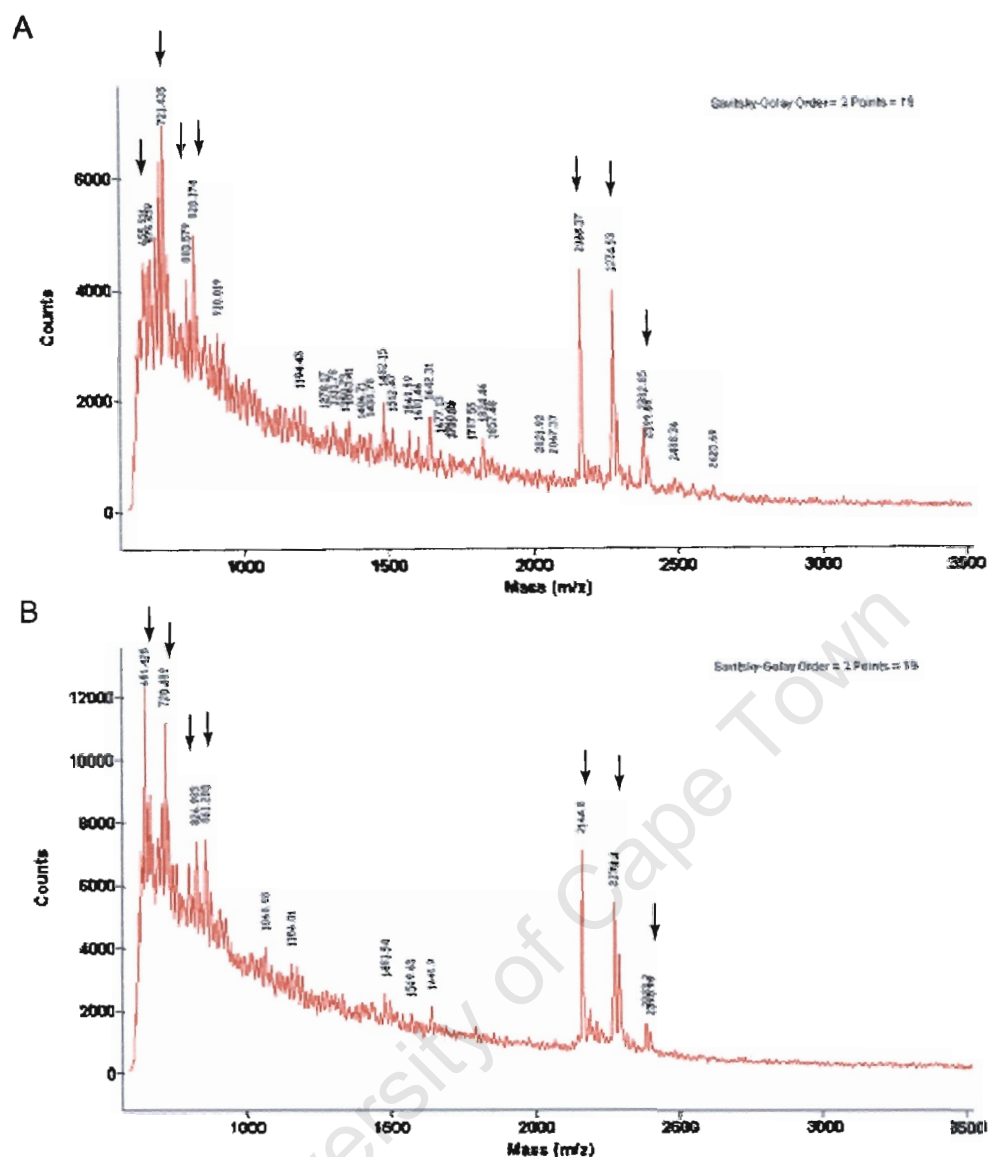


Figure 3.6. The MALDI-TOF spectra of a partial tryptic digest of protein band 1 excised from the 1 day (A) and 6 day (B) affinity protein fractions resolved by SDS-PAGE electrophoresis.

3.7. Identification of the second band

The analysis of the partial tryptic digests of the proteins present in gel slice 2 (see Figure 3.5) with the default MS-Fit settings did not uniquely identify any *S. cerevisiae* proteins (see Figure 3.7). The use of a lower stringency setting, requiring only three peptide peak matches to the database, identified a carboxykinase or a phosphatase, or a cell-wall interacting protein, like ankyrin or kinesin. There was no clear match to Hho1p, and we concluded that Hho1p was not present in gel slice 2. We note that the increasing amplitude of peaks towards to lower mass region of the spectrum could indicate non-specific proteolysis during the isolation of the proteins from the gel slice, which was expected to compound the analysis.

3.8. Identification of the third band

A comparison of the mass peaks present in the mass spectra of the partial tryptic digests of the proteins in gel slice 3 to a database of theoretical masses of *S. cerevisiae* proteins (Figure 3.8) gave conclusive results for both the one day and the six day cultures. Heat shock protein 70 (Ssa3p), with a molecular weight of 70.5kDa predicted from the sequence, and kinesin-like protein 3 (Kip3p), with a predicted molecular weight of 91.1kDa, were both identified with the default stringency settings in the one day sample. This result confirmed that the size range of the proteins that we analysed was expected to contain the c-myc tagged Hho1p.

An analysis of the gel slice protein sample from the six day culture identified a diphosphate synthase and a signalosome protein. Neither the one day nor the six day sample contained the c-myc tagged Hho1p.

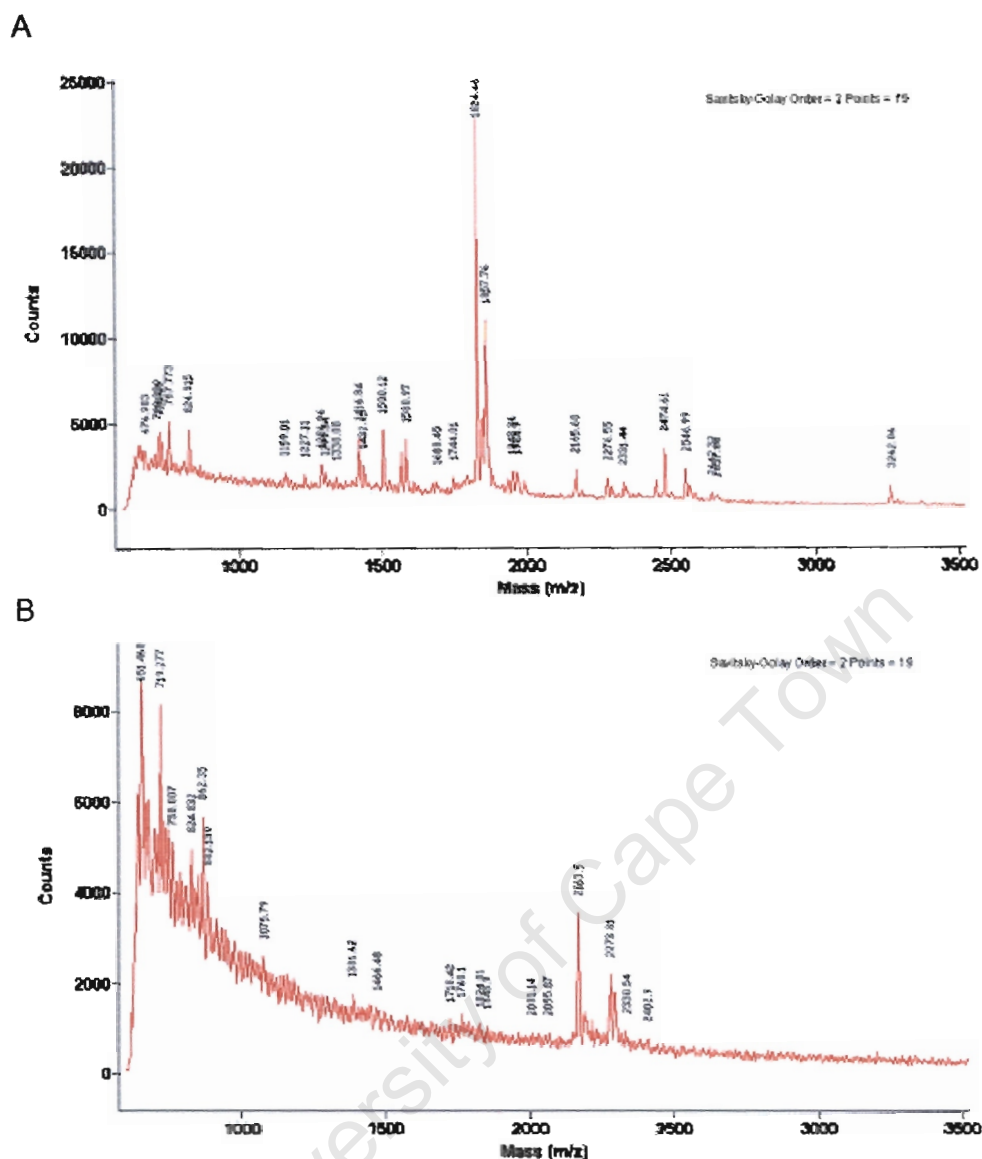


Figure 3.7. The MALDI-TOF spectra of a partial tryptic digest of protein band 2 excised from the 1 day (A) and 6 day (B) affinity protein fractions resolved by SDS-PAGE electrophoresis.

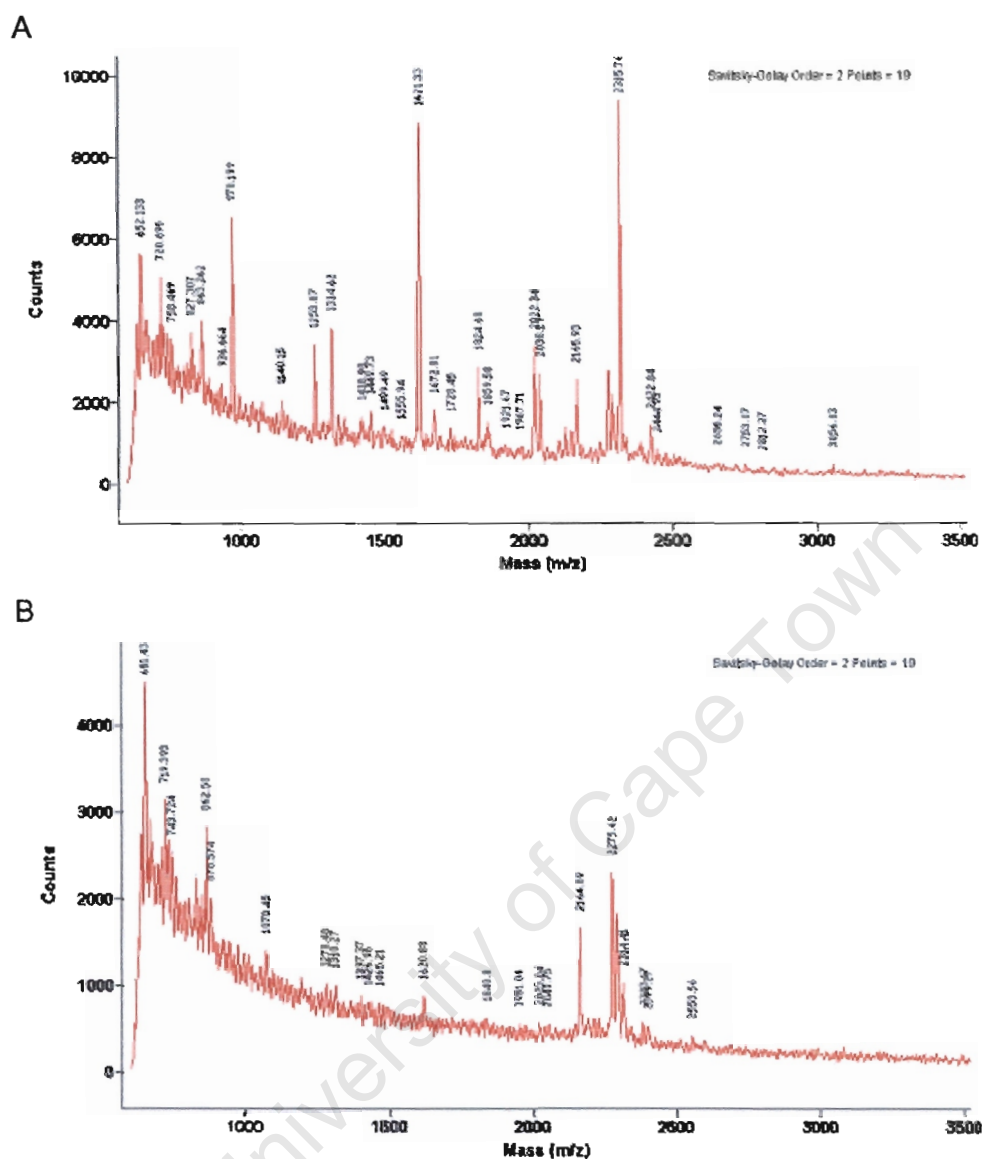


Figure 3.8. The MALDI-TOF spectra of a partial tryptic digest of protein band 3 excised from the 1 day (A) and 6 day (B) affinity protein fractions resolved by SDS-PAGE electrophoresis.

3.9. Identification of the fourth band

The analysis of the mass spectra of the partial tryptic digests of the proteins isolated from band 4 (Figure 3.9) identified adenosuccinate lyase in the one day sample, and Pib2p, a protein that binds phosphatidylinositol-3-phosphate, in the six day culture. There was no clear match to Hho1p in this sample.

3.10. Perchloric acid extraction of Hho1p

It was previously reported that Hho1p was present at a level of approximately 13% that of the core histone H2B (Downs et al., 2003). Hho1p was therefore expected to be an abundant protein, and the absence of the protein in all four gel slices was surprising. However, it was also reported that the isolated globular domain two of Hho1p unfolded spontaneously with time (Ali and Thomas, 2004), a physical property that could influence the protease susceptibility of Hho1p. It is noteworthy that original attempts to isolate linker histones from *S. cerevisiae* did not succeed, and may have been due to ready proteolysis of this protein during isolation. We therefore investigated an alternative method to the affinity chromatographic purification of the protein from total protein extracts, in an attempt to procure sufficient quantities of Hho1p for the MALDI-TOF analysis.

The perchloric acid extraction has been used for purifying linker histones from mammalian (Kratzmeier et al., 1999), murine (Deterding et al., 2004) and *Xenopus* cells (Cuisset, Tichonicky, and Delpech, 1999). We tried to increase the yield and purity of the recovered sample using the method used to extract human linker histone variants published by Doenecke and co-workers (Albig et al., 1998).

The protein samples recovered by perchloric acid extraction of the one day and the six day cultures were electrophoretically resolved by SDS-PAGE (Figure 3.10). Referring to this Figure, it is clear that the perchloric acid extraction did

not give samples highly enriched for a single protein, unlike the results obtained with mammalian tissues Kratzmeier et al., 1999). We do, however, note the presence of a strong band at approximately 70kDa, the region where c-myc tagged Hho1p was reported to migrate (Downs et al, 2003), suggesting that perchloric acid extraction may be a suitable method to isolate Hho1p from *S. cerevisiae*. The further exploration of perchloric acid extraction as a method to recover *S. cerevisiae* Hho1p is planned for the future.

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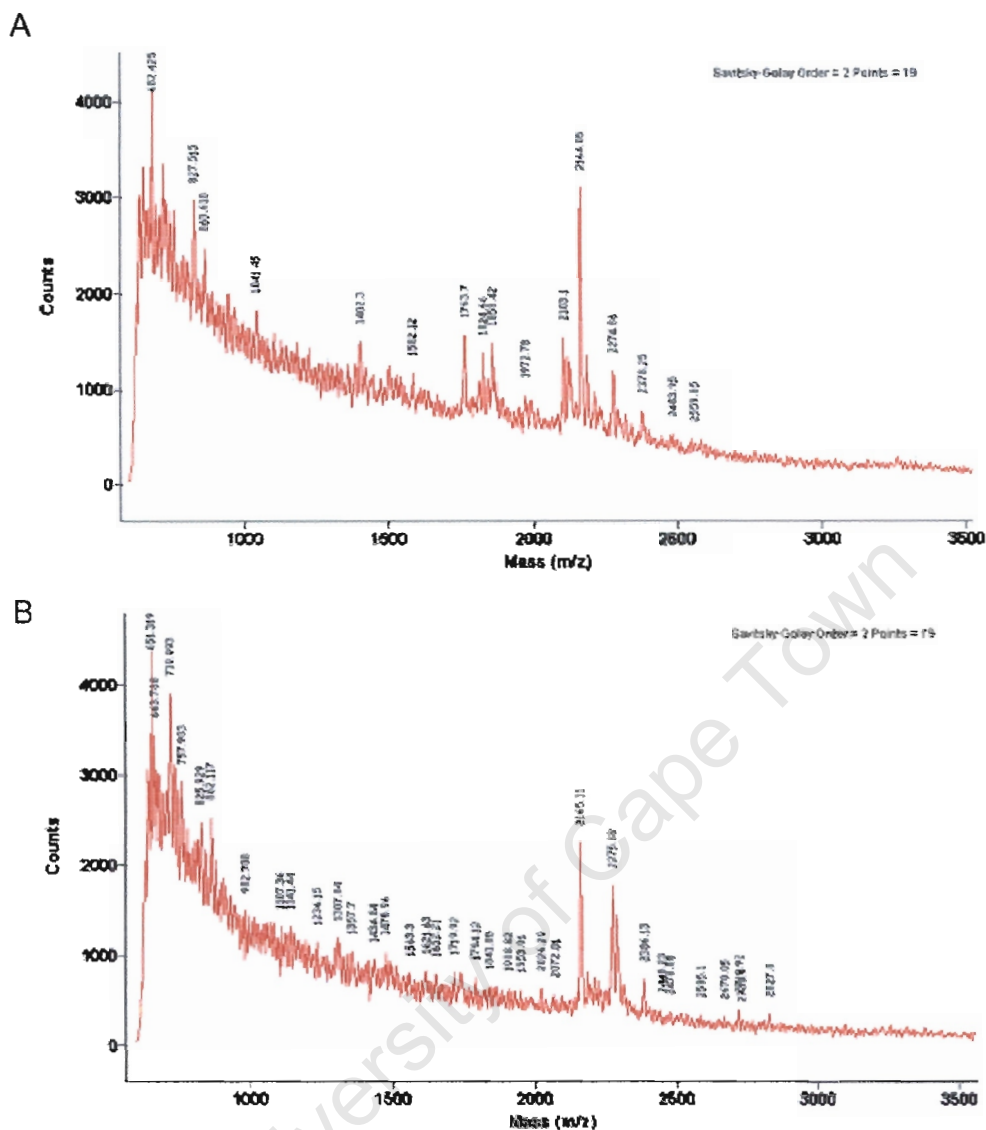


Figure 3.9. The MALDI-TOF spectra of a partial tryptic digest of protein band 4 excised from the 1 day (A) and 6 day (B) affinity protein fractions resolved by SDS-PAGE electrophoresis.

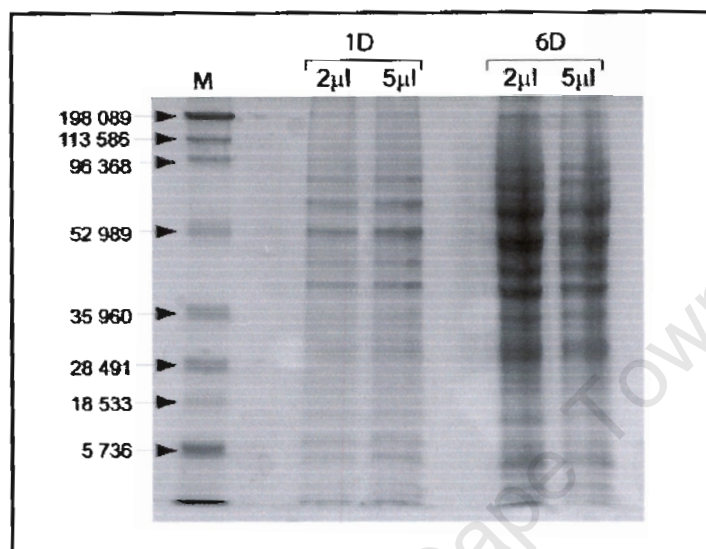


Figure 3.10. Perchloric acid extraction of *S. cerevisiae* cultures. Aliquots (2μl and 5μl) of perchloric acid extracted *S. cerevisiae* cultures grown for one day (1D) or six days (6D) were resolved on a 12% (w/v) SDS-PAGE gel. A Coomassie blue stain of the gel is shown.

Chapter 4

Conclusion and Discussion

4.1 A model for H1 regulation of DNA function

It was recently shown in our group that the linker histone Hho1p in *Saccharomyces cerevisiae* preferentially bound to chromatin in stationary phase (Schafer *et al.*, manuscript in preparation). It was also reported that phosphorylation of H1 increased dynamic exchange of the linker histone in chromatin (Contreras *et al.*, 2003), that the H1-like proteins in *Tetrahymena* became dephosphorylated during starvation (Dou *et al.*, 2005), and that H1 inhibited chromatin remodeling *in vitro* (Horn *et al.*, 2002). Taken together, these results suggested that H1 may become phosphorylated to increase dissociation of H1 from chromatin, and that this dissociation was required for other covalent histone modification enzymes and ATP-dependent remodeling activities to modify the chromatin to a structural state that allowed processes such as transcription, replication, recombination and DNA repair. The increase in binding of Hho1p to chromatin in yeast stationary phase therefore suggested that the phosphorylation state of Hho1p may change during entry and exit of stationary phase, and that this change is a requirement for chromatin decondensation and subsequent participation of the DNA molecule in the various genetic processes in which it is required. A hypothetical regulatory model, involving phosphorylation of histone H1, is proposed in Figure 4.1.

4.2 Is Hho1p phosphorylated?

In this study we investigated possible changes in the phosphorylation state of specifically serine and threonine residues during stationary phase entry and exit. Although a clear phosphorylation signal was found for both residue types, we could not detect a significant and reproducible change in phosphorylation state

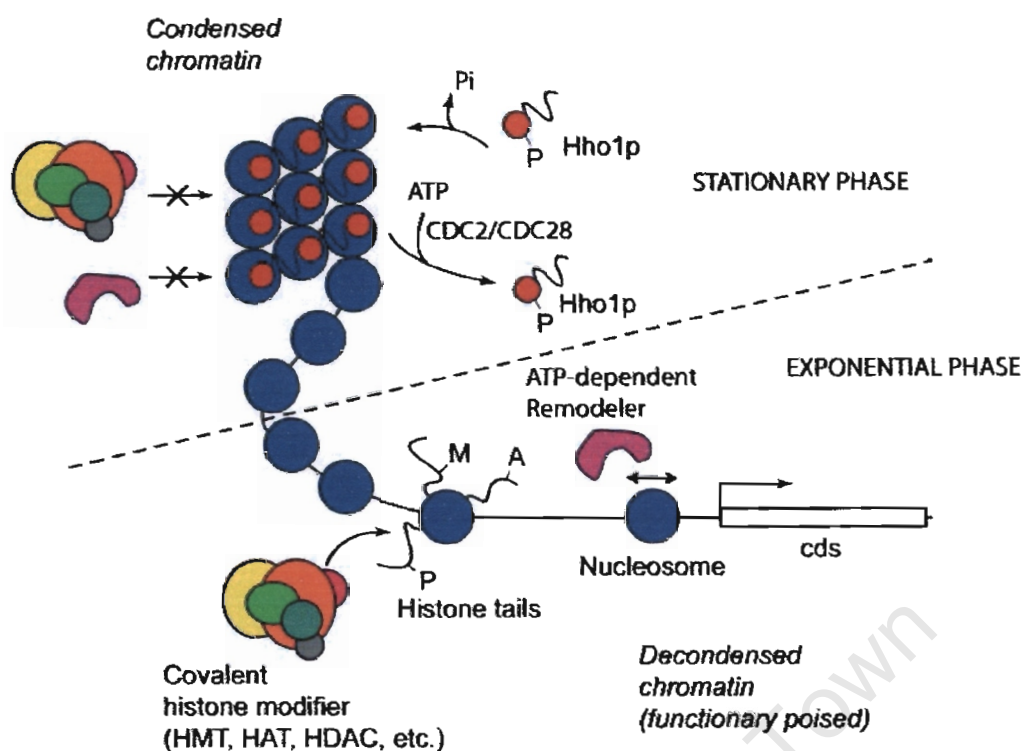


Figure 4.1. The regulation of chromatin decondensation by histone phosphorylation in stationary and exponential phase in *Saccharomyces cerevisiae*. The dephosphorylation of H1 allows binding to chromatin, stabilizing the higher-order condensed structures of chromatin. For decondensation to occur, the CDC2/CDC28 mediated phosphorylation of H1 is required. It is likely that this decondensation is accompanied by covalent modification of core histone tails, such as acetylation of the histone H4 N-terminal tail, implicated in chromatin condensation. This decondensed state characterises chromatin in exponentially growing yeast cells, where further covalent modifications and chromatin remodeling can occur to allow DNA transcription, replication, repair and recombination. Histone H1 dissociation may thus be an early event required for the transition from stationary to exponentially growth phase in *Saccharomyces cerevisiae*.

corresponding to the quasi-senescent state of yeast. This finding suggested that

- either serine and threonine was not phosphorylation targets in stationary phase,
- only a minor proportion of serine and threonine residues changed their phosphorylation state, which would not have been detectable by the antibody hybridisation approach,
- that other residues such as tyrosine was a major

target of such regulated phosphorylation, or iv), that other types of modifications was responsible for the increased binding of Hho1p in stationary phase.

To address these latter two possibilities, we attempted to isolate Hho1p from exponentially growing cells and from cells in stationary phase, to determine the covalent modification of all residues in the protein by MALDI-TOF mass spectrometry. This technique was previously successfully used to identify covalent modifications of core histones and linker histones.

Although we could immunoprecipitate Hho1p, and isolate the protein by antibody affinity chromatography, the yield and purity was not sufficient to arrive at an unambiguous answer using MALDI-TOF. Our inability to prepare homogenous samples of Hho1p in sufficient yield may be related to the unique *S. cerevisiae* protein. It was recently reported that the second globular domain of Hho1p spontaneously unfolded under conditions where the first globular domain of Hho1p, and the single globular domain of metazoan linker histones, remained fully folded (Ali and Thomas., 2004). The structural instability of the second globular domain may be due to the presence of short α -helical regions between the consensus α -helices 1 and 2 of the single winged helix fold (Ali and Thomas, 2004). In this regard, yeast H1 may be a protein that only becomes fully folded in association with DNA. It is possible that in the partially unfolded state, particularly during the isolation procedure, the yeast Hho1p protein is highly susceptible to proteolysis. It may be for this reason that we could not identify the Hho1p in the molecular weight range on the SDS-PAGE gel where it was expected to resolve. Our ability to visualize c-myc tagged Hho1p at the expected position indicated that some of the protein, at least, remained intact, and migrated as a full length molecule.

We conclude that although a major change in the phosphorylation state of the serine and threonine residues does not seem to correspond to entry and exit of stationary phase, it remains possible that phosphorylation of only one or a few

residues regulate H1. It is also a formal possibility that the binding of Hho1p is not regulated by the modification of the linker histone, but that modification of the core histones increase/decrease affinity of Hho1p for the nucleosome. In this regard, the competition reported between the binding of the core histone H3 N-terminal tail in the linker DNA region and histone H1, is relevant. For instance, it is possible that the N-terminal tail of H3 becomes acetylated during growth to stationary phase, weakening the histone-DNA interaction, and allowing H1 to successfully compete for binding in the linker DNA region. We also note that it is possible that H1 is loaded onto chromatin by an unidentified, specialized H1 assembly factor. Assembly factors are involved in the deposition of core histones onto newly replicated DNA, as well as the swapping of histone isotypes during transcription. It is therefore possible that the activation of such a linker histone assembly factor is required to load H1 onto chromatin.

4.3 Future directions

It is clear from the work reported here that the increase in binding of Hho1p to chromatin in stationary phase is not due to the phosphorylation of a single or a few serine and threonine residues. To address the possibility that the phosphorylation state of only one or a few residues compared to many phosphorylated residues, changed, a detailed mass spectrometric analysis will be necessary. Such an approach will also identify other potential modified residues during passage to and from stationary phase. In order for such a mass spectrometric study to be undertaken, the isolation of sufficient quantities (a few micrograms) of pure H1 will be necessary. In this study, such an isolation proved problematic. We therefore foresee that a major effort must be made to understand the difficulty behind isolation of the yeast H1 compared to the H1 from the tissues of other species. If the mass spectrometric study does not provide evidence of any modifications synchronous with entry and exit of stationary phase, the possibility that the modification of core histones regulate H1 binding must be investigated. This will require the straightforward isolation of

core histones from exponential phase and stationary phase by RP-HPLC, and the mass spectrometric analysis of the four core histones. An alternative idea, the activation of an unidentified linker histone assembly factor, can be explored by a detailed proteomic analysis of partner proteins that are co-immunoprecipitated with linker histone Hho1p. Such activities are planned for the future.

University of Cape Town

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